THE CONSTITUTION OF PROTAGON AND ITS ESTIMATION IN BRAIN,

BY

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COMPOSITION and ESTIMATION of PROTAGON.

Since Liebreich (1865) obtained from brain tissue this substance to which he gave the name Protagon, much has been written of its composition. But what has been written is of such a contradictory nature that it is difficult from an examination of the literature of the subject to say what precisely is the composition of this much discussed substance. In fact so very contradictory are the statements made that one is almost led to believe that it is quite impossible that the same subject is under discussion on all occasions. For instance some authors state that protagon contains a certain amount of potassium, others deny that it contains any; some say that the melting point is indefinite, others that it is definite; on the one hand it is held to be a mechanical mixture, on the other that it is a chemical compound.

From the evidence even to date it is difficult to decide which authors have been right and which wrong in their statements regarding protagon. To give some idea of the present confusion we may first quote Halliburton (1907): "It has now been conclusively proved in confirmation of what Thudichum stated in 1874 that protagon is a mixture of phosphorised and non-phosphorised substances in such proportion that it usually contains about one per cent of phosphorus".

More recently, Leathes (1910) says: "Protagon therefore is supposed to be a single complex combination of all the chemical/

chemical compounds that can be demonstrated in nerve tissues, a combination that is in the same class with "protoplasm" and as little capable of chemical definition". It is possible to have some sympathy with Halliburton's statement but the second quotation shows either an ignorance of the literature or represents the extent of confusion to which one may be led by a study of what has been written on this subject. Halliburton's statement is based, speaking generally, on the work principally of Hoppe Seyler, Thudichum, and after them of Gies, and Rosenheim and Tebb.

On the other hand we have Gamgee followed by Baumstark, Ruppel, and Cramer.

As representing the second point of view take Cramer (1908): "Protagon is a substance of definite chemical composition retaining this composition after repeated crystallisation". When such conflicting statements are possible there is, obviously, room for more work on protagon.

As a result of the prolonged controversy we have positive evidence that protagon consists of

Cerebrosides and Sphingomyelin.

Now the constitution of cerebrosides is known. There remains sphingomyelin, of whose constitution there is still room for doubt. According to Thierfelder, cerebrosides are not decomposed by baryta so that the decomposition products of protagon by baryta hydrolysis must be due to sphingomyelin. Of these, cholin has been determined by Cramer. Sphingosin and fatty acids/

acids of sphingomyelin have been demonstrated by Thierfelder (1906). There remains, presumably, an unknown alcohol. Thudichum (1901) states that glycerin is absent from sphingomyelin. From Liebreich onwards it has been generally understood that protagon contained glycerophosphoric acid. Since sphingomyelin is the only phosphorus containing complex isolated by protagon decomposition, if glycerophosphoric acid is present in protagon it ought to be found in sphingomyelin. It is intended as a continuation of the present work on protagon to try to clear up this difficulty; but the evidence, though so far not quite complete, points to the correctness of the view held for so long - that protagon does contain glycerophosphoric acid.

While we have positive evidence that protagon consists of cerebrosides and sphingomyelin, yet the question as to whether these substances form a chemical compound or a mechanical mixture has been and still is the subject of much controversy.

P A R T I.

Comparison of the molecular weight of Protagon with the molecular weights of the Phosphatide and Cerebrosides prepared from it.

The following observations were carried out with the object of obtaining fresh information on this and other points.

Part I. Introductory.

All authors are agreed that it is possible to prepare from protagon two cerebrosides, namely cerebrin and homocerebrin (phrenosin and kerasin of Thudichum: 1901), and a phosphatide, sphingomyelin. But while, according to Hoppe Seyler, Thudichum and their followers, protagon is simply a mixture of these three substances, together perhaps with a number of other simpler substances, the followers of Gamgee (1880) maintain that protagon contains these substances in chemical combination, which is easily broken up with the liberation of the constituent phosphatide and cerebrosides. Hitherto all attempts to decide this question have been made by studying the behaviour of protagon on the one hand and of cerebrin, homocerebrin and sphingomyelin and mixtures of the three on the other hand, towards certain solvents such as alcohol, chloroform, pyridine, and others. But this line of argument has failed to bring about an agreement. Thus Thudichum and Rosenheim and Tebb (1910) state that protagon has an indefinite and variable melting point, and completely alters its composition on re-crystallisation from large volumes of alcohol; in other words its behaviour is that of a mixture of a phosphatide and cerebrosides. On the other hand Gamgee, Roscoe, Baumstark, Ruppel and Cramer find that protagon has a definite melting point and retains its composition on recrystallisation from large and small/

small volumes of alcohol; in other words, its behaviour, according to these authors, is not that of a mixture of a phosphatide and cerebrosides. There is therefore hardly any common ground on which a discussion as to the nature of the substance in question is possible along these lines. Indeed, as has been said, the only conclusion an impartial observer can draw is that the substance investigated by Thudichum and his followers is not identical with the one studied by Gamgee and his followers. Nor are these contradictions to be found only between the statements of those who attack and those who defend the existence of protagon as a definite compound. The observations of Posner and Gies that protagon retains its composition when dissolved in warm alcohol and cooled immediately, however large the volume of alcohol, is diametrically opposed to the statements of Rosenheim and Tebb and yet these authors quote each other in support of their views.

A new line of argument is necessary in order to settle this question. It is offered in Part I of this thesis and is as follows:-

The chemical composition of cerebrin and homocerebrin is fairly well known owing mainly to the work of Thudichum, Kossel and Freytag (1893), Thierfelder, and Levene and Jacobs (1912).

Thierfelder (Kitagawa and Thierfelder, 1906), from his observations on the products of the acid hydrolysis of cerebron, which is either identical with or closely allied to cerebrin, has calculated the formula C_{48} H_{93} O_9 N which has also been accepted/

accepted by Levene and Jacobs (1912) as the result of their analysis. The molecular weight of cerebron, according to this formula would therefore be 827. In the case of the closely allied homocerebrin the molecular weight has been determined directly by means of the elevation of the boiling point by Kossel and Freytag and was found to lie between 945 and 1027. In the case of sphingomyelin our knowledge depends mainly on the statement of Thudichum, who from a study of the products of hydrolysis gives to it the formula $C_{52} H_{104} O_9 N_2 P$. The molecular weight of sphingomyelin would be, accordingly, 931.

The three substances which can be obtained from protagon, therefore, have molecular weights of such an order of magnitude that, if they follow Raoult's law, a definite elevation of the boiling point should be noticeable in solutions of moderate concentrations (3 per cent to 5 per cent) of these substances, especially if one uses a solvent with a relatively high constant, such as chloroform. One gram of a substance with the molecular weight of 1000, would, in a 4 per cent solution in chloroform, elevate the boiling point by about 0.10°. If therefore protagon were a mixture of these substances it should, with the concentrations given, produce a distinct elevation of the boiling point of chloroform.

On the other hand, if protagon contained these substances in chemical combination it would have a molecular weight at least approximating to 3000.

It/

It may, of course, be considerably higher; in fact Cramer calculated from the amount of sulphur in protagon a molecular weight of 5778. If therefore protagon were a combination and not a mixture of cerebrosides and phosphatide the boiling point of a 3 per cent to 5 per cent chloroform solution of protagon would show either no elevation at all of the boiling point of chloroform or only a very slight one compared with that produced by cerebrin, homocerebrin, sphingomyelin or mixtures of these substances.

Observations on the boiling point of chloroform solutions of these substances by means of Beckmann's method ought therefore to give a decisive answer to the question whether protagon is a chemical combination or a mixture of phosphatide and cerebrosides. And since with substances of high molecular weights the determination of the molecular weights can only be approximate, it may be pointed out that this answer is not dependent on the exact numerical evaluation of the molecular weights nor upon slight quantitative differences between them, but upon differences of such an order of magnitude as to be qualitative.

EXPERIMENTAL.

For this work protagon was prepared by a method similar in essentials to that of former workers and particularly to that employed by Cramer and Wilson. As there were some variations in manipulation, the method is given here.

Ox brain after having been freed from blood and membrane as/

as far as possible and put through a mincing machine, was placed in a wide mouthed stoppered bottle with sufficient cold acetone to cover it. The bottle was shaken for several hours. The material was then drained through a coarse French filter paper on a Buchner funnel attached to a suction pump. This operation was repeated three times to thoroughly free the brain from water and cholesterin. Replacing acetone by cold ether the same process was repeated in order to free from lecithin and cephalin. The material was then washed, while on the filter, with cold alcohol to free entirely from ether solution. (The ether solution was retained for future work on brain "lecithin".) The material was replaced in the bottle and absolute alcohol which had been heated to boiling was poured over it. The bottle was shaken by hand for a couple of minutes and the material transferred to a hot water filter funnel. Two alcohol extracts were made. The filtrate was received in an ice-cooled vessel. Protagon separated out as a white flocculent precipitate with a distinctly crystalline appearance. The protagon was twice re-crystallised from small quantities of boiling alcohol. When dried in a vacuum desiccator over sulphuric acid the protagon appeared as a white pulverulent powder. No more than ten ox brains were used at any one time as it is not easy to manipulate larger quantities. Fresh ox brains were similarly treated till about 20 gms. of pure protagon had been obtained. The treatment with warm alcohol was carried out as quickly as possible since, according to various authors, prolonged/

prolonged treatment with warm alcohol may cause decomposition.

Identification of Protagon.

It was considered sufficient for identification to find the melting point and the phosphorus and nitrogen contents.

Phosphorus and nitrogen were estimated by Neumann's and Kjeldahl's methods respectively. The melting point was taken in the usual way except that the heating medium (sulphuric acid) was raised to a temperature approximating 200° before the capillary with protagon was inserted. This was done to avoid the decomposition which various authors have noted as taking place about 190° and upwards. Protagon melted sharply at the recorded temperature.

The values approximate closely to those of such observers as Gamgee, Roscoe, Baumstark, Rupper, Gies, and Cramer.

Table I.

Identification of Protagon.

Melting point.	Phosphorus content. (per cent)	Nitrogen content. (per cent)
205°	.99 (average of two estimations.)	2.1 (average of two estimations.)

Comparing this table with the records of former observers it is noticed that -

(1) The melting point is rather higher than any yet recorded. This is probably due to some extent to the manipulation mentioned.

(2)/

(2) The phosphorus content approximates as will be seen to the usual one per cent associated with protagon - and which Halliburton considers fortuitous.

(3) The nitrogen content is slightly less than that of most workers - 2.3 to 2.5 being about usual percentage.

The results shown in this table (I) are, however, sufficiently accurate for our purpose - to obtain the same substance as has been dealt with by the majority of former authors.

Part of the protagon obtained in this way was used for the preparation of cerebrin and homocerebrin, by boiling it with baryta water. This method, which was used originally by Parcus and by Kossel and Freytag, has been re-introduced recently by Lorrain Smith and Mair (1910) and by Loening and Thierfelder (1911).

Protagon is made into a fine emulsion with saturated baryta water, heated under a reflex condensor in a vigorously boiling water bath for one hour and the mixture filtered. The residue, after boiling with a mixture of alcohol and acetone, is repeatedly extracted with boiling acetone, from which on cooling a mixture of cerebrin and homocerebrin separates out. The mixture thus obtained was recrystallised twice from boiling acetone and was obtained eventually in the form of a white powder more granular in appearance than protagon. Since it was intended to compare the behaviour of protagon with that of a mixture of the cerebrosides which can be prepared from it, the separation of the two cerebrosides was not carried out. The observations were made by Beckmann's method with an apparatus having an electrical heating/

heating device. The amount of chloroform used was measured in every case by volume, not by weight, and amounted to 25 c.c. The chloroform was "chloroform from chloral" except in one case when "chloroform from acetone" was used. In almost every case a reading of the boiling point of the pure solvent was taken both before and after the experiment in order to correct for changes due to variations in atmospheric pressure. The boiling point of a solution was taken as the point where three successive readings at intervals of five minutes gave constant results. The observations made with protagon and with a mixture of cerebrin and homocerebrin respectively are recorded in Tables II and III. These observations show clearly that the size of the protagon molecule is very much larger than that of the molecules of cerebrin and homocerebrin. Indeed the protagon molecule is so large that even in concentration exceeding 5 per cent it fails to produce an elevation of the boiling point of chloroform. Now it is possible to calculate from the amount of galactose liberated from protagon, cerebrin, and homocerebrin respectively that <u>one gram</u> of protagon contains 0.6 to 0.7 gram of cerebrosides and 0.3 to 0.4 gram of sphingomyelin. Even if one assumed that sphingomyelin had a very much larger molecule than the data of Thudichum would indicate, or if one assumed that for some reason or other it did not follow Raoult's law, perhaps by aggregating in solution with a colloidal form - even then the elevation of the boiling point produced by <u>one gram</u> of protagon ought to be at least as great as that produced by .6

to/

to .7 gram of the cerebrosides obtained from it. As a matter of fact, however, 0.7 gram of a mixture of cerebrin and homocerebrin is sufficient to produce a distinct elevation of the boiling point of 25 c.c. of chloroform, while even double the quantity of protagon is incapable of producing that effect.

Table II.

Boiling point of chloroform solutions of protagon.

No. of Experiment.	Volume of chloroform in c.c.	Weight of substance in solution. in gms.	Boiling point.	e.	Barometer.
I.	25	0	59.62	-	734
	"	0.6664	59.62	0	"
	"	0	59.62	-	"
II.	25	0	59.92	-	742
	"	0.9730	59.92	0	"
	"	0	59.92	-	"
III.	25	0	60.50	-	Not read.
	"	0.9430	60.45	-0.05	"
	"	1.5920	60.45	-0.05	"
	"	0	60.50	-	"
IV.	25	0	59.62	-	734
	"	.6664 (protagon) +	59.62	0	"
	"	0.4240 (naphthalene) +	59.99	+0.37	"
	"	0.4306 (naphthalene)	60.36	+0.37	"
	"	0	59.62	-	"
Molecular weight of naphthalene calculated 119,121 (128)					

Table III.

Boiling point of a chloroform solution of a mixture of cerebrin and homocerebrin.

No. of Experiment.	Volume of chloroform in c.c.	Weight of substance in solution. in gms.	Boiling point.	e.	Barometer
V.	25	0	59.50		728
	"	0.7305	59.53	+0.03	"
	"	1.5140	59.63	+0.13	"
	"	0	59.50		"

It follows, then, that the observations recorded in Tables II and III are incompatible with the view that protagon is a mixture of sphingomyelin, cerebrin and homocerebrin. It will be noted that the elevation of the boiling point produced by cerebrin and homocerebrin increases with increasing concentration out of proportion to the amount of substance added. This irregularity has no bearing on the problem under discussion but it is of some general interest since a similar phenomenon has been observed in the case of other substances with large molecules, for instance in the case of colloidal solutions of silicic acid, tungstic acid and molybdic acid. It will also be noted in one case (Experiment No. III) that the boiling point of protagon instead of remaining constant showed a slight diminution, which, however, was not increased on adding more protagon. Since in this case a less pure sample of chloroform ("chloroform from acetone") was used, the irregularity may probably be referred to this fact.

Although/

Although a chloroform solution of protagon shows no elevation of the boiling point, yet the presence of protagon does not hinder the boiling point of chloroform being affected by addition of another substance. This is shown by the fact that addition of a weighed quantity of naphthalene to a chloroform solution of protagon produced an elevation of the boiling point commensurate with its molecular weight. In order to compare the molecular weight of sphingomyelin with that of cerebrin, homocerebrin and protagon, sphingomyelin was prepared by heating protagon with pyridine to 50° for twenty minutes. The precipitate which falls out on cooling the pyridine is removed by filtration. After washing with cold alcohol and drying in vacuo it had a phosphorus percentage of 2.3 per cent; it represents, according to Rosenheim and Tebb (1910) sphingomyelin with a slight admixture of cerebrosides. The pyridin filtrate from sphingomyelin when poured into an excess of acetone gives a precipitate consisting of cerebrosides with a slight admixture of sphingomyelin. The sphingomyelin obtained in this way was not freed from its slight admixture of cerebrosides since the object of these observations is to obtain comparative values for protagon on the one hand and the substances or mixtures of the substances which can be obtained from it, on the other.

The observations made with sphingomyelin prepared in this way are given in Table IV. It will be seen that sphingomyelin produces a distinct elevation of the boiling point of chloroform, thus indicating that the size of the molecule of sphingomyelin is/

is of about the same order of magnitude as that of cerebrin and homocerebrin and very much smaller than that of protagon. The observations with sphingomyelin, therefore, confirm the conclusions arrived at from the observations with cerebrin and homocerebrin.

Table IV.

Boiling point of chloroform solution of sphingomyelin (with slight admixture of cerebrosides).

No. of Experiment.	Volume of chloroform in c.c.	Weight of substance in solution.	Boiling point.	e.	Barometer.
VI.	25 "	0	60.56 60.60	- +0.04	754 "
VII.	25 "	0 1.0948	60.35 60.42	 +0.07	752 "

Table V.

Boiling point of a chloroform solution of a mixture of sphingomyelin, cerebrin and homocerebrin.

No. of Experiment.	Volume of chloroform in c.c.	Weight of substance in solution.	Boiling point.	e.	Barometer.
VIII.	25 "	0 1.0948 (sphingomyelin) + 0.7210 (cerebrosides) + 0.7196 (cerebrosides) 0	60.35 60.42 60.45 60.47 60.25	- +0.07 + +0.03 + +0.02	752 749

The preparation of sphingomyelin and the cerebrosides from protagon/

protagon by heating the latter with pyridin made it possible to compare protagon with a mixture of sphingomyelin and cerebrosides in approximately the same proportions in which they can be obtained from it. For that purpose the chloroform solution of sphingomyelin obtained in Experiment No. VII, Table IV, was used. After having obtained a constant value for the boiling point of this solution the cerebrosides obtained by the pyridin treatment described above were added. The results are given in Table V. Experiment No. VI, Table IV, was made on the forenoon of the same day. Experiment No. VII, Table IV, and the observations recorded in Table V on the afternoon of that day. It will be seen that the Barometer fell from 754 mm. at 9 a.m. to 749 mm. at 3 p.m. There is, of course, a corresponding fall of the boiling point of the pure solvent. Since in the observations recorded in Table V the boiling points of three different solutions were determined, one hour and a half elapsed between the beginning and the end of these observations, and even during that time a distinct fall in the boiling point of the pure solvent was noticed. Although the barometric variations would tend to diminish any elevation in the boiling point produced by the addition of cerebrosides, there is nevertheless clear evidence of a rise in the boiling point of the chloroform solutions of sphingomyelin in every addition of cerebrosides. Taking the last observation, it will be seen that a mixture of about 1.1 gram sphingomyelin and 1.4 gram cerebrosides, having a phosphorus percentage of 1 per cent corresponding/

corresponding to that of protagon, produced a total rise of 0.22°. Of this rise 0.07 is due to the sphingomyelin present and consequently a rise of 0.15 is due to the cerebrosides. This is approximately the same value as that obtained before in the observation recorded in Table III. In other words, the effect produced by a mixture of sphingomyelin, cerebrin and homocerebrin is simply the additive effect produced by the substances constituting the mixture. If protagon were a loose molecular compound which could be reconstituted by simply mixing these substances together, as has been suggested by some writers, the addition of cerebrosides to the solution of sphingomyelin should diminish the elevation of the boiling point produced by sphingomyelin. As a matter of fact the opposite takes place. The observations show then, again, that protagon is not a mixture of sphingomyelin, cerebrin and homocerebrin but that it must contain these substances in chemical combination. They also show that it is not possible to reconstitute protagon by mixing together in certain proportions the cerebrosides and phosphatides which can be prepared from it.

P A R T II.

Estimation of Protagon in Total Lipoids of Brain by Sulphur Content.

Part II. Introductory.

By the evidence of the workers mentioned and the results of the investigations now given protagon must be given its true position as a constituent of brain tissue. By those opposed to the idea that protagon is a chemical entity and a proximate constituent of brain it has been considered to be made up of lecithin and cerebrin, a phosphatide and a cerebroside, and in order to get the cerebrosides completely freed from the phosphatide these workers subjected protagon to repeated washing with ether - but in vain. They were never able by this means to rid the "cerebrin" of the last traces of "lecithin". Although obtained by this treatment, cerebrin (or cerebron) was looked upon as a proximate constituent of brain and methods were adopted by Koch and others for its estimation. With protagon in its true position as a chemical compound found along with other lipoids in brain tissue it is necessary to consider methods for its estimation, and since certain methods have been employed to estimate its constituent cerebrosides, knowledge of these will be of value in discussing methods for the estimation of protagon. Methods have been suggested or employed by previous workers and it is intended here to subject these to re-examination.

For instance it has been suggested by Cramer that if protagon be the only sulphur-containing lipoid in brain, it would be possible to estimate the amount of protagon by finding the sulphur content of the total lipoids of brain. No proof has, however/

however, been brought forward that protagon is the only sulphur containing lipoid in brain nor for that matter has there been given any definition of what is meant by a lipoid. Leathes says "The term lipoid was first used by Overton, perfectly justifiably, without any chemical connotation for substances resembling in their solubilities the fats . . . . . It may be, and commonly is, used to include anything soluble in ether or even alcohol". Protagon is not soluble in cold ether or in cold alcohol but it so closely resembles in chemical composition substances which are recognised as lipoids that it would be absurd not to extend such a general term to include protagon. If we take a general fat solvent such as chloroform, we find that this is capable of dissolving ordinary ether soluble lipoids as well as protagon, so that we might define the lipoids of brain as those constituents of brain which are soluble in chloroform. If this is done and chloroform used to extract the total lipoids of brain we find that under these conditions protagon is not the only sulphur containing substance derived from brain so that if we include all the chloroform extractions of brain as lipoids, then we cannot estimate protagon by the sulphur content.

If, however, we treat brain with acetone and ether (cholesterin could be estimated by Windaus' method and the soluble phosphatides by phosphorus content) then by taking a chloroform or chloroform-methyl alcohol extract of the remainder, the/

the sulphur content of this would represent the amount of protagon.

EXPERIMENTAL.

For the purpose of testing the possibility of estimating protagon by the sulphur content of total lipoids, about 50 gms. of fresh brain tissue were taken. After being thoroughly stirred with formaldehyde it was spread out on plates and dried at first at room temperature, finally in incubator.

The dry powder was thoroughly exhausted with chloroform in a Soxhlet apparatus. The chloroform extract was evaporated at room temperature. The sulphur contents of pure protagon (as prepared in Part I) and of the total lipoids were taken. On comparing these the sulphur content of the total lipoids (chloroform extract) was found to be higher than that of pure protagon, so that it was seen that some sulphur containing substance was present besides protagon. For the estimation of sulphur in both cases it was found, after an examination of various methods, that the most preferable was the method employed by Benedict, quoted in the 3rd edition Hawk's Practical Chemical Physiology, for estimation of sulphur in urine. As this may not have been used previously, apart from use with urine, it may be given here.

Estimation of sulphur.

1 Gram of material was rubbed up with a few drops of Benedict's solution (a mixture of copper nitrate and potassium chlorate)/

chlorate) into a paste, more solution was added until an emulsion was obtained. To this was added 20 c.c. of the solution and the contents of the mortar poured into a silica crucible, capacity about 40 c.c. This was heated very gently at first and then more strongly, the gas being kept regulated so that the contents of the crucible were gently boiling. The emulsion was added in two parts, the crucible never being more than half full. This precaution with that of gentle heating prevented loss by spitting. The heating of the crucible was continued till the contents solidified, fused and solidified a second time. The flame was now raised in two stages and finally the dry, black, contents of the crucible were heated for ten minutes. About 10 c.c. of 25% HCl were added. This gave a clear green solution with a few copper oxide specks. This was filtered into an Ehrlenmeyer flask (250 c.c.) and 100 c.c. cold water added. 10 c.c. of 10% $BaCl_2$ solution were added drop by drop. After being allowed to stand all night (Benedict says one hour) the contents of flask were filtered through a weighed Gooch filter. A "blind" test ot Benedict's solution showed absence of sulphur.

Table VI.

Comparison of sulphur content of pure protagon with that of total lipoids (chloroform extract of dry brain).

Weight of substance in gms.	Barium sulphate on Gooch in gms.	S.factor.	Percentage of.S.
1 (Protagon)	.0350	.137	.49
2 (chloroform extract)	.1522		1.05

Table VII.

Sulphur content of chloroform methyl-alcohol extract of brain, after previous extraction with acetone and ether.

Weight of substance in gms.	Barium Sulphate on Gooch in gms.	S.factor.	Percentage of S.
1	.0310	.137	.42

Discussion of Part II.

It will be seen from the results given in Table VI that it is impossible to estimate protagon in brain by taking the sulphur content of a chloroform extract of brain since the chloroform extracts another sulphur containing substance which, according to Levene, though not a lipoid as regards composition, is like these substances as regards solubility. By freeing the brain of this substance - and at the same time removing cholesterin and lecithin both of which may be easily estimated - protagon, being the only sulphur containing lipoid now left, may be estimated by the sulphur content.

Owing to the presence of this other sulphur containing substance and also to the fact of sulphur being present in protagon in very small amount, it would not then be advisable to employ this method of estimating protagon.

To estimate protagon it is necessary to find and consider some constituent which is not present in any other lipoid and an investigation of this matter is made in Part III of this thesis.

P A R T III.

Estimation of Protagon in Total Lipoids of Brain by Acid Hydrolysis.

Part III. Introductory.

Noll (1899) estimated protagon in brain tissue by the amount of galactose split off. This method is fallacious if, as Gamgee held, there is other cerebroside in brain besides the protagon cerebrosides. It was for the purpose of testing the correctness of Noll's figures that this part of the work was undertaken. The question to be answered is this: Is there any cerebroside in brain besides that in protagon? The answer is to be found in a comparison of the ratio of the total sulphur to total galactose in pure protagon with that of the ratio of total sulphur to total galactose in an extract of brain which contains no sulphur other than protagon sulphur and yet contains all the cerebrosides, protagon and otherwise. Such an extract is to be found, as shown in Part II, by exhausting brain with acetone and ether and then taking a chloroform-methyl extract. Acetone and ether remove water cholesterin, lecithin and cephalin, as well as all sulphur containing material extra to protagon, leaving behind protagon and all other cerebroside material. A chloroform-methyl alcohol extract of this will contain, then, protagon and free cerebroside if any.

It is found that the ratio of sulphur to galactose in free protagon is the same as that of sulphur to galactose in the chloroform-methyl alcohol extra, showing that there is no cerebroside other than that in protagon, and thus proving that Noll's figures are correct. The somewhat easy decomposition of protagon, probably accounts for Gamgee's error.

EXPERIMENTAL.

Pure protagon as prepared in Part I was taken. Fresh ox brain was washed with acetone and ether as described in preparation of protagon (Part I). The remaining material was now treated with chloroform-methyl alcohol (3:1). The solution was evaporated at room temperature. Sulphur was estimated in protagon and "extract" by Benedict's method, galactose by acid hydrolysis. The method of sulphur estimation has already been described. For the estimation of galactose the material was hydrolysed for 24 hours with dilute hydrochloric acid (Koch, 1904). The sugar was estimated by Bertrand's method, in preference to that of Koch, who weighed the copper.

Table VIII.

The ratio of sulphur to galactose in protagon compared to the ratio of sulphur and galactose in chloroform-methyl alcohol extract of brain (after exhaustion with acetone and ether).

	Weight of substance in gms.	Copper in mgms.	Galactose in mgms.	Percentage of Galactose.
(1) Protagon	.2	44.9	23.8	11.9
(2) "Extract"	.2	39.78	21.0	10.1

Protagon Sulphur = .49%
(Table VI)
" Galactose = 11.9%

"Extract" sulphur = .42%
(Table VII)
" Galactose = 10.1%

$$\frac{.49}{11.9} = .041$$

$$= 1 : 1$$

$$\frac{.42}{10.1} = .041$$

Discussion of Part III.

To estimate protagon it is advisable to use some constituent which is not present in any other lipoid. Gamgee, with whose work otherwise on protagon much of this thesis will be found in agreement, was under the impression that in brain tissue there existed cerebroside material other than protagon cerebroside. Had this been so then protagon could not have been estimated by the total galactose split off as representing the only cerebroside material. Since, as Part III of this thesis shows, there is no other cerebroside in brain lipoids, protagon may be estimated by splitting off galactose and we conclude then that Noll's figures are correct. This estimation according to Koch takes a considerable time. This question leads to the next question - that of rate of hydrolysis of protagon.

P A R T IV.

Rate of Acid Hydrolysis of Protagon.

Part IV. Introductory.

Koch considered that galactose could only be completely split off from nervous tissue by somewhat prolonged hydrolysis and he usually boiled the material with dilute acid for twenty hours. Since, accordingly, the estimation of protagon would be a somewhat lengthy process, it was considered of some importance to find the actual rate of hydrolysis of protagon by boiling with dilute acid to see if a shorter time would not suffice.

The following figures will clearly show that in much shorter time than Koch considered necessary, the hydrolysis is complete. Since only the rate of hydrolysis is required, the amount of galactose split off is not given.

EXPERIMENTAL.

The weighed quantity of protagon (previous workers have obtained best results by using small quantities) was rubbed up in a mortar with a few drops of dilute hydrochloric acid (1%) until a paste was formed, more dilute acid was then added until an emulsion was obtained. This is done to prevent or lessen, as far as possible, the frothing which usually takes place while the mixture is being boiled. In each case (Table IX) .2 gms. was taken. The emulsion was poured into long necked, round bottomed, flasks. Material left clinging to the inside of the neck of the flask on filling was washed down with more acid (100 c.c. of acid in all was used). The flasks were placed under a reflex condenser on/

on wire gauze over a Bunsen flame, and the contents allowed to boil gently.

Two flasks were heated at a time. The whole of the contents of each was removed and the estimations made at the intervals stated. As has been said above, a certain amount of frothing usually takes place during boiling and particles of material are apt to be stranded on the sides of the flask. This can almost entirely be eliminated if the following precautions be taken:-

(1) Make a good emulsion.

(2) Add a few drops of alcohol, to lessen surface tension.

(3) Heat very gently until the mixture has been boiling for some time.

(4) Shake the flask gently from time to time.

As it was found somewhat difficult to regulate the heating a different plan of heating was afterwards adopted.

The work shown in Table X was carried out as follows:- .1 gm. of protagon was with 10 c.c. of dilute acid (5%) placed into each of six small, wide mouthed, flasks (about 50 c.c. capacity). To each was attached a 2 ft. air condenser. The flasks were half immersed in a boiling water bath and removed at the times stated. In both cases (Tables (IX & X) the filtering of the solution was aided by the addition of sodium sulphate, a clear filtrate being obtained.

Table IX/

Table IX.

Rate of hydrolysis of protagon by boiling with dilute (one per cent) hydrochloric acid.

Weight of Protagon in gms.	Time.	Copper in Gooch crucible, in gms.
.2	2 hours	.0088
.2	4 hours	.0150
.2	6 hours	.0348
.2	8 hours	..0346

Table X.

Rate of hydrolysis of protagon by boiling with dilute (5 per cent) hydrochloric acid.

Weight of Protagon in gms.	Time	Potassium Permanganate (Bertrand's solution No. 4. in c.c.
.1	1 hour	.5
.1	2 hours	1.6
.1	3 hours	2.1
.1	4 hours	2.5
.1	5 hours	2.5
.1	6 hours	2.5

Discussion of Part IV.

The result of the investigation undertaken in this part of the thesis was successful in showing that the estimation of protagon by the splitting off of galactose by acid hydrolysis can be completed in a very much shorter period than has been considered possible. Koch's lengthy hydrolysis along with his method of weighing the copper, made the whole process of estimating cerebroside material occupy considerable time. It has now been shown here that the hydrolysis is complete in about/

about 5 hours. The estimation being completed by Bertrand's volumetric method shortens the time for estimation very considerably.

The fact that combined cerebroside was here being subjected to acid hydrolysis suggested the interesting question as to whether the cerebroside being so combined would yield its sugar less quickly than free cerebroside. Naturally one would think that the cerebroside in a chemical compound would have its galactose split off less readily than if it (the cerebroside) were free.

Thus by comparing the rate of hydrolysis of combined and free cerebroside we should probably learn something of the condition under which cerebroside is present in protagon. This is dealt with in Part V.

P A R T V.

Rate of Acid Hydrolysis of Protagon after previous saponification with Baryta.

Part V. Introductory.

When protagon is boiled with concentrated baryta water, according to various writers, it yields certain decomposition products. These are, most likely, glycerophosphoric acid, cholin, and fatty acids - the glycerophosphoric acid forming barium glycerophosphate and the fatty acids soaps. Now according to Thierfelder, cerebrosides are not decomposed by baryta so that what decomposition takes place must be of the sphingomyelin. Whatever the ultimate products of decomposition it will be readily granted that the first action of the baryta must be to split up the protagon into its constituent cerebrosides and sphingomyelin. Whatever the subsequent fate of these substances, it was thought it would be interesting to discover whether previous saponification with baryta altered the rate of acid hydrolysis.

If protagon were a mixture of cerebrosides and phosphatide then the rate of acid hydrolysis would not be affected by previous saponification, for the cerebrosides in a mixture would be just as free for acid hydrolysis whether previous saponification had taken place or not since, as has been stated above, cerebrosides are not acted on by baryta. If, on the other hand, protagon is a chemical compound, then saponification will certainly free the cerebrosides and leave them to be more readily hydrolysed by acid. If it can be shown, then, that the initial hydrolysis after saponification is greater than the initial hydrolysis without previous saponification, we must infer that protagon is a chemical compound.

EXPERIMENTAL.

Protagon was rubbed up in a mortar with concentrated baryta water until a fine emulsion was formed. This was placed in the small flasks as described in Part IV. The flasks were placed in a water bath kept gently boiling for four hours. The baryta was first neutralised and then dilute hydrochloric acid (5%) was added. Acid hydrolysis was carried on, the flasks being removed at the times stated and the rate of hydrolysis taken. The result is shown in the following table. It may be mentioned that similar figures were obtained by a slightly different procedure. Two flasks (250 c.c., long necked, round bottomed), A and B were taken. 1 gm. of protagon as a baryta emulsion was placed in each. The emulsion in A was now neutralised. The contents of both flasks were now boiled for four hours. At the end of this time the emulsion in B was neutralised. Dilute acid was now added to both flasks the greatest care being taken that the contents of each should have not only exactly the same degree of acidity but also of volume of material. When this was adjusted, the flasks were set in a boiling water bath. It may be mentioned here that in almost every instance the bath used for experiments in this work was electrically heated and the boiling was very constant and satisfactory. As has already been mentioned, some frothing takes place and of course this is especially troublesome in the case of saponification and to this fact must be attributed the lower rate of hydrolysis.of the saponified material after the initial/

initial hydrolysis. It simply amounts to this, that in the case of the previously saponified material, owing to loss, there is less material to hydrolyse.

This diminution in the rate of hydrolysis as could easily be seen by tracing the graphs of the two rates is only apparent. As a matter of fact it strengthens our inference from the results we have obtained, namely, when protagon has been previously saponified, the initial rate of acid hydrolysis is greater.

From both flasks, equal aliquot parts were removed and the rate of hydrolysis taken.

Table XI.

Comparison of rates of acid hydrolysis of protagon with and without previous saponification with baryta.

Weight of Protagon in gms.	Time.	Potassium permanganate in c.c.	Potassium permanganate in c.c. (Previously saponified.)
.1	1 hour	.5	1.2
.1	2 hours	1.6	1.3
.1	3 hours	2.1	1.7
.1	4 hours	2.5	1.7

Discussion of Part V.

The results obtained in this part of the work are of the highest importance and form a strong corroboration of what we have stated in our discussion of Part I, namely, that protagon is a chemical compound of cerebrosides and phosphatide.
The inference from Part V is quite clear. If the cerebroside in protagon were free, one would expect that its initial rate of hydrolysis would be greater than if it were combined. Now the/

the initial rate of acid hydrolysis of the cerebroside of protagon without previous treatment is less than the initial rate after such treatment as sets the cerebroside free. Therefore it is surely not too much to assume that the cerebroside of protagon is not free.

GENERAL SUMMARY.

Certain writers, notably Hoppe Seyler, Thudichum and in recent years Posner and Gies, Rosenheim and Tebb, supported by Halliburton and others, have contended that protagon is a fortuitous mixture of cerebrosides and phosphatides. Others, and notably Gamgee and Cramer, have maintained that protagon is a chemical compound of cerebrosides and a phosphatide.

In the present work in Part I and Part V, by two new methods, proofs are brought forward which support the statements of the latter group of workers, that protagon is a chemical compound of the phosphatide (sphingomyelin) and cerebrosides (cerebrin and homocerebrin). This being so, Thudichum's view that phosphatides and cerebrosides were the most complex forms of lipoids present in tissues must be incorrect. Phosphatides contain phosphorus but no galactose, cerebrosides contain galactose but no phosphorus. Now protagon contains both galactose and phosphorus and since the cerebrosides preponderate it seems quite convenient to refer to protagon as a "phospho-cerebroside" as suggested by Cramer (1911).

In Part II the method suggested by Cramer (1911) of estimating protagon in total lipoids of brain by the sulphur content is subjected to examination and it is shown that if by total lipoids we mean a chloroform extract then we cannot estimate protagon by the sulphur content since a chloroform extract contains another sulphur containing substance besides protagon/

protagon. If, however, brain be treated with acetone and ether, thus removing known lipoids such as cholesterin, lecithin and cephalin which may be estimated, besides the sulphur containing substance mentioned, we can estimate protagon by taking the sulphur content of the remaining material.

In Part III the question of estimating protagon in brain by the amount of galactose split off is discussed. The point at issue is this: Is there any cerebroside in brain other than protagon cerebrosides? The answer is found by a comparison of the ratio of sulphur to galactose in brain tissue from which all sulphur containing material extra to protagon has been removed, while at the same time retaining all the cerebroside material, protagon and otherwise, with the ratio of sulphur to galactose in pure protagon. The answer is that there is, in brain, no cerebroside other than those in protagon and therefore protagon may be estimated by the splitting off of galactose. Noll's figures are, therefore, correct.

Part IV deals with the rate of acid hydrolysis of protagon and the result simply amounts to this - that it is not necessary, as Koch did, to treat brain tissue for more than 5 - 6 hours to complete the hydrolysis.

Part V, although like Part IV in dealing with rate of hydrolysis, must be taken along with Part I as affording another proof that protagon is a chemical compound and not a mixture of cerebrosides and a phosphatide. If protagon were a mixture then/

then saponification by baryta previous to acid hydrolysis - since cerebrosides are not affected by baryta - ought not to affect the initial rate of hydrolysis. Since the initial rate is increased by previous saponification, we have here a strong proof that protagon is a chemical compound.

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